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Sample Collection and Preparation Methods Affecting Mutagenicity and Cytotoxicity of Coal Fly Ash

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16. ABSTRACT

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SAMPLE COLLECTION AND PREPARATION METHODS AFFECTING MUTAGENICITY AND CYTOTOXICITY OF COAL FLY ASH

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INTRODUCTION

Reports by several investigators describing the biological activity of coal fly ash have presented a variety of results which in some cases (Fisher et al., 1979; Clark and Hobbs, 1980; Kubitschek et al., 1980; Mumford and Lewtas, in press) are conflicting. The biological activity of coal fly ash may differ because of one or or more of the following factors: (1) the samples studied were from different sources; (2) the samples were prepared for bioassay differently; (3) the sampling method differed, and, therefore, collected samples were different in chemical or physical properties which affect the biological activity. Several variables involved in coal fly ash studies -- source, sample collection and preparation methods, bioassay method -- are undoubtedly responsible for the diversity of biological effects observed. The objectives of this study were to examine the sample preparation and collection factors which may affect the observed biological activity caused by coal fly ash and to evaluate the mutagenicity and cytotoxicity of fluidized-bed combustion (FBC) fly ash from experimental and commercial units. The bioassays used in this study were the Ames Salmonella plate incorporation test for mutagenicity and the rabbit alveolar macrophage (RAM) system for cytotoxicity.

Worldwide use of coal as an energy source is predicted to increase substantially in the next two decades. The burning of coal by conventional combustion (CC) methods presents a number of environmental problems. Moreover, the addition of new control methods, such as flue gas scrubbing, may not always be economical in conventional plants. Therefore, it has been of great interest to develop alternative coal combustion technologies that are environmentally acceptable and economically feasible.

Of the new coal technologies under development, FBC is closest to large-scale commercialization. A limited number of commercial FBC units are now available. A commercial unit currently in operation at Georgetown University, Washington, DC, was the source of several of the emissions reported here. FBC systems for the production of steam or electricity have several advantages over CC systems:

(1) High heat transfer coefficients and volumetric heat release rates permit a reduced boiler size, which, in turn, lowers capital cost.

(2) Limestone is used as the bed sorbent material to remove SO_2, thus reducing SO_2 emissions and permitting use of high sulfur coal.

(3) The lower FBC temperature range (800 to 900°C, vs. the CC range of 1400 to 1600°C) can decrease NO_x emissions (Fennelly et al., 1977).

The low FBC combustion temperature, however, may decrease the combustion efficiency and increase the emission of mutagenic organics. Kubitschek et al. (1980) and Clark and Hobbs (1980) have reported mutagenic effects of coal fly ash from experimental FBC units. It has been speculated that the lower FBC combustion temperatures increase the emission of polycyclic organic matter (Kubitschek and Williams, 1980).

SAMPLE PREPARATION STUDIES

Two fly ash samples -- one from an FBC source and a reference sample from a CC source -- were used to study sample preparation methods for the mutagenicity bioassay. The CC fly ash sample was collected from a conventional coal-fired power plant burning Alabama Eastern bituminous coal with 1 to 2% sulfur and 11% ash. The combustion temperature was about 1400°C. The sample was collected downstream of an electrostatic precipitator (ESP) by the fabric filter of a high-volume particulate sampler. Sample

collection temperature was ~ 132°C. The fly ash particle size was, in general, < 3 μm, with a geometric mean diameter of 1.05 μm. The FBC fly ash sample was collected from a pressurized FBC miniplant (combustor diameter was 33 cm) at Exxon burning Pennsylvania Eastern bituminous coal with 2% sulfur and 8% ash. The combustor temperature was about 870°C. The sample was collected at ~ 165°C by a fabric filter after three stages of conventional cyclones. A detailed description of the Exxon FBC miniplant and the sample collection has been reported (Kindya et al., 1980). The particle size was < 3 μm with a geometric mean diameter of 0.54 μm.

Mutagenicity Studies

Two sample preparation methods were examined for Salmonella mutagenicity assay: direct testing of particles and testing of the extractable organics. The Ames Salmonella typhimurium plate incorporation method (Ames et al., 1975) with the following modification was employed. The standard minimal histidine was added to the plating media instead of the soft agar overlay. The plates were incubated 72 h (Claxton et al., 1979). For metabolic activation a 9000-g supernatant (S9) of a liver homogenate was prepared using Arochlor 1254-induced male rats (Charles River CD 1; Ames et al., 1975). Negative and positive control tests were conducted with each experiment. A sample was considered positive if it showed a maximum response two times greater than the spontaneous rate and if it gave a positive, linear, dose response. The linear portion of the dose-response curve was used to calculate a linear regression line. The slope of the linear regression was reported as revertants per weight unit of samples tested. Figure 1 presents a comparison of the mutagenicity of FBC and CC fly ash samples, based on experiments in which the particles are placed directly in agar overlay. The FBC fly ash particles without metabolic activation gave a dose response for mutagenic activity, whereas CC fly ash showed negative response with and without activation. With activation, FBC fly ash also showed a negative response for mutagenicity. The direct testing of fly ash particles indicated the bioavailability of mutagens and required much smaller sample size. This method, however, was less sensitive than solvent extraction prior to bioassay.

For solvent extraction studies, sonication and Soxhlet extraction methods using a series of the organic solvents with different polarity were examined to determine the most efficient methods in removing the mutagens from the FBC and CC fly ash samples. The four solvents examined were cyclohexane, dichloromethane, acetone, and methanol, with polarity indices of

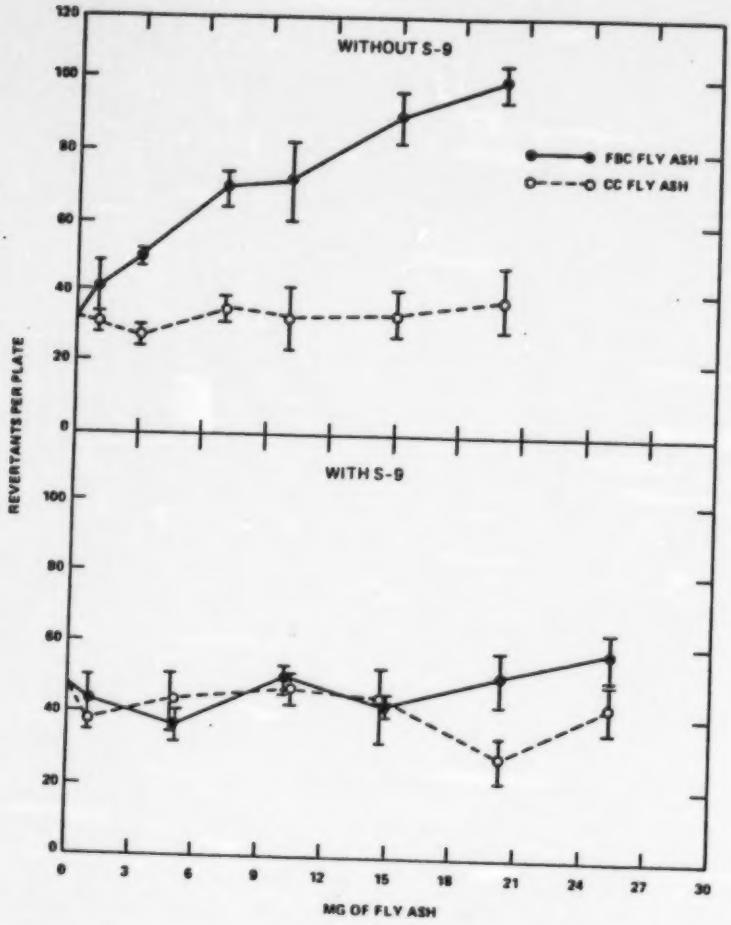

Figure 1. Mutagenicity of fly ash particles in Salmonella typhimurium TA98. Data presented are means ± standard deviation of three replicates.

0.0, 3.4, 5.4, and 6.6, respectively. The detailed extraction methods were reported previously (Mumford and Lewtas, in press). The results of the solvent extraction studies for percent of extractable mass are shown in Figure 2. These data show that Soxhlet extraction and sonication yielded comparable extractable mass for each solvent. Also shown is that the more polar solvents acetone and methanol extracted more mass; extraction with the less polar solvents cyclohexane and dichloromethane yielded lesser

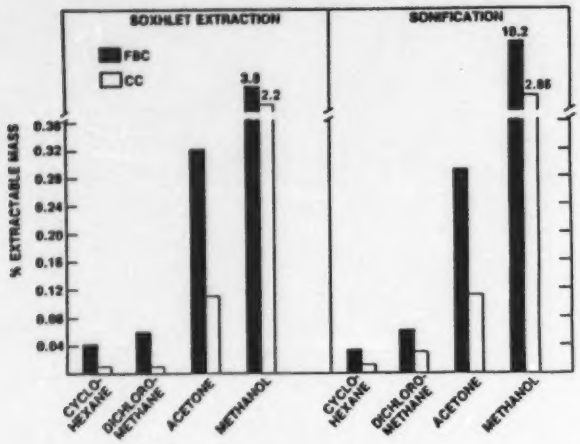

Figure 2. Percent of extractable mass.

amounts of extractable mass. The FBC fly ash sample consistently contained more organic solvent extractable mass than CC fly ash, even though the FBC fly ash collection temperature was 33°C higher than the CC fly ash collection temperature.

The mutagenicity of the organic extracts of fly ash, determined with Salmonella typhimurium TA98 without S9 activation, is shown in Figure 3. Dichloromethane extraction resulted in the highest mutagenic activity per unit of extractable mass, followed by acetone and cyclohexane. The methanol extractable material produced a negative response in mutagenicity tests. These data show that this FBC fly ash sample has higher mutagenic activity than the CC fly ash sample for all the solvents which removed mutagens.

In Table 1, mutagenicity of the fly ash sample is expressed as revertants per microgram of extractable mass and as revertants per milligram of equivalent fly ash. Since acetone extraction yielded a relatively high extractable mass and moderate mutagenicity, this extraction gave the highest number of revertants per milligram of equivalent fly ash. These data show that addition of S9 generally decreased the mutagenic activity. This indicated the presence of direct-acting mutagens in both fly ash samples.

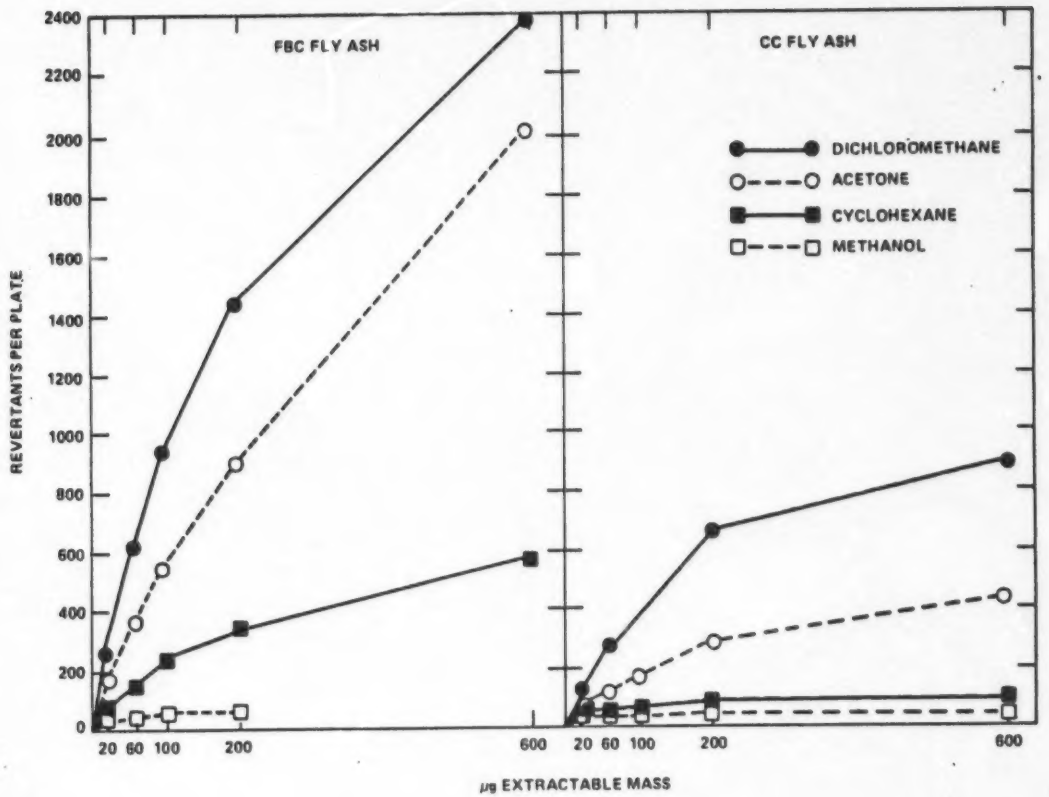

Figure 3. Mutagenicity of organic extracts of coal fly ash in Salmonella typhimurium TA98 without metabolic activation. Each point is an average of two replicates.

Table 1. Mutagenicity of Fly Ash in the Salmonella typhimurium Plate Incorporation Assay Using TA98

Sample	Extraction Solvent	% Extractable Mass	Revertants per microgram extractable mass[a]		Revertants per milligram equivalent fly ash[b]	
			With Activation	Without Activation	With Activation	Without Activation
FBC fly ash	Dichloromethane	0.06	5.21	9.43	3.13	5.66
	Acetone	0.30	1.33	4.45	3.99	13.35
	Methanol	7.0	neg	neg	neg	neg
	Cyclohexane	0.04	0.87	1.71	0.35	0.68
	None	--	--	--	neg	3.32[c]
CC fly ash	Dichloromethane	0.02	--	3.07	--	0.61
	Acetone	0.11	0.43	1.34	0.47	1.47
	Methanol	2.55	neg	neg	neg	neg
	Cyclohexane	0.01	--	0.10	--	0.01
	None	--	--	--	neg	neg

[a]Revertants per microgram of organics obtained by computing the slope of the linear regression in the linear portion of the curve.
[b]Revertants per milligram equivalent calculated by multiplying revertants per microgram of organics times the number of microgram organics per milligram of fly ash.
[c]Revertants per milligram of fly ash obtained by computing the slope of the linear regression from the testing of particles directly.

Since methanol extraction resulted in high yield of mass but showed no mutagenic activity, elemental and anion analyses were conducted to determine if methanol removed primarily inorganic matter. Indeed, a considerable amount of inorganics, especially the sulfates, was extracted by the methanol, as shown in Table 2. The methanol extract also showed a lower percentage of carbon in comparison to the acetone extract, which would indicate low organic content. The low organic content and the high inorganic matter of the methanol extract may account for the negative mutagenic response.

Cytotoxicity Studies

The alveolar phagocytic macrophages have been generally considered essential for defending the host against inhaled particles and other toxicants. The rabbit alveolar macrophage (RAM) system was used to evaluate the cytotoxicity of fly ash particles in this study. The technique for the assay has been reported previously (Mumford and Lewtas, in press). The RAM cells were exposed to FBC or CC fly ash particles for 20 h at 37°C *in vitro*. At the end of the incubation period, the cytotoxicity indices of viability, viability index, adenosine triphosphate, and protein synthesis were evaluated. The results are shown in Figure 4. These data show that FBC and CC fly ash samples both exerted dose-response toxic effects on the RAM cells. The FBC fly ash sample appears to be more cytotoxic than CC fly ash sample.

Table 2. Elemental and Anion Analysis

Sample	Extractable Mass (%)	C (%)	H (%)	F^- (µg/g)	Cl^- (µg/g)	$SO_4^=$ (µg/g)
FBC fly ash						
Acetone extract	0.30	26.3	5.3	1.9	485.0	61.7
Methanol extract	7.0	5.1	3.7	178.3	672.7	3110.4
CC fly ash						
Acetone extract	0.11	38.3	5.9	0.8	22.6	12.7
Methanol extract	2.55	3.2	4.4	120.5	38.9	5777.7

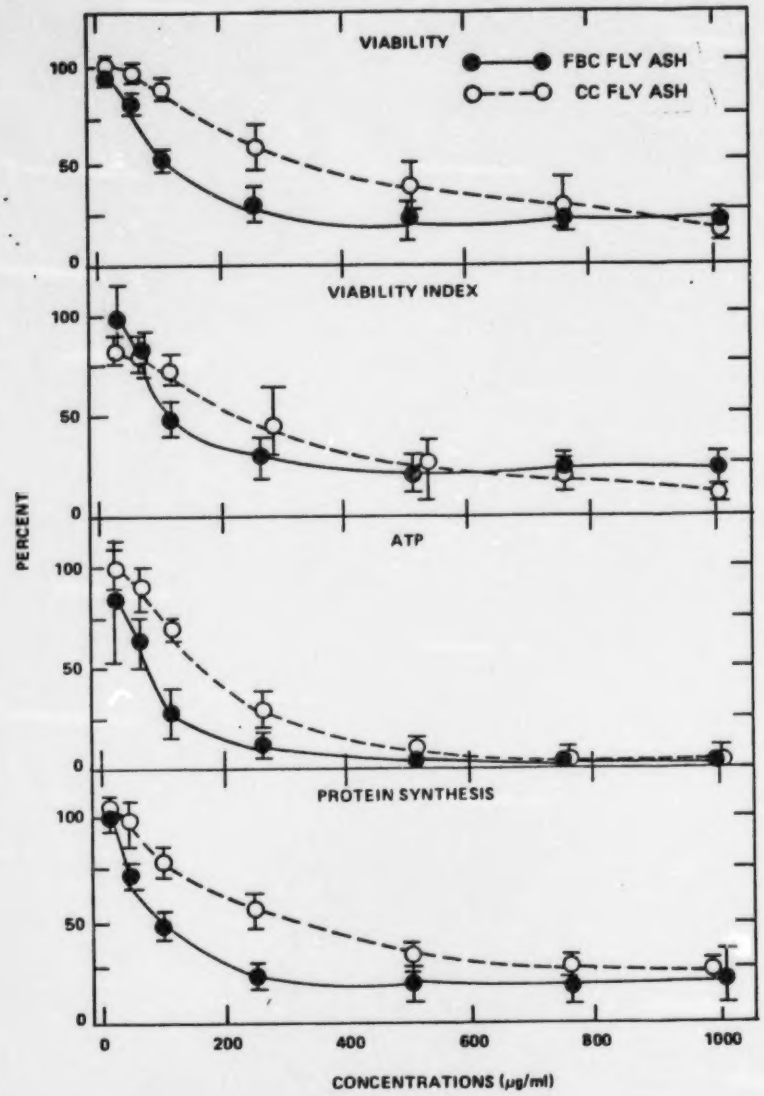

Figure 4. Toxic effects of fly ash on RAM cells after a 20-h exposure in vitro. Data presented are means ± standard deviation of three experiments (nine replicates). Viability is calculated and expressed in percent viable cells. Viability index was obtained by multiplying cell viability by total cell numbers as a fraction of the control. Adenosine triphosphate, ATP (fg/ml), and protein synthesis (disintegrations per minute) are expressed as percent of control.

SAMPLE COLLECTION STUDIES

The three devices most commonly used to remove particles from gas effluents are the cyclone, the electrostatic precipitator (ESP), and the baghouse filter. These collectors are based on different principles for removing particles from the air. Cyclones collect particles by centrifugal separation. Baghouse filters collect particles by forcing the gas effluent through the filters and trapping the solids. An ESP collects particles when gas effluent containing the particles passes through the device's electric discharge area, where ionization of the gas occurs. The ions produced will collide with the suspended particles, causing an electric charge within the particles. These charged particles drift toward an electrode with the opposite charge and are deposited on the plates. These different collection or sampling methods, therefore, result in the selective collection of particles with particular properties, which may produce different biological activity in bioassays.

At the Exxon FBC miniplant, the effluent stream passes through three stages of high efficiency, conventional cyclones in the plant and then through the ESP or baghouse filter outside the plant. Four FBC fly ash samples collected by the two cyclones (secondary and tertiary cyclones), the fabric filter, and the ESP at various temperatures were examined and compared for particle size, toxicity, and mutagenicity. The collection temperatures were 830°C for the secondary cyclone, 650°C for the tertiary cyclone, and 170°C for the filter and for the ESP. The median volume diameters of the four fly ash samples were 4.6, 3.0, 1.7, and 1.5 μm for the secondary cyclone, tertiary cyclone, ESP, and filter samples, respectively. Figures 5 and 6 present the results of cytotoxicity studies with the RAM assay and mutagenicity studies using the Salmonella assay, with and without activation. These data show that the two cyclone samples, collected at higher temperatures, contained larger particles which were very weakly cytotoxic or mutagenic. The filter and ESP samples, collected downstream of the cyclones at lower temperatures, contained finer particles that showed higher cytotoxic and mutagenic activity in the test systems. The ESP sample was the most mutagenic.

Biological activity vs. sample collection temperature was plotted (Figure 7), and, at lower collection temperatures, greater cytotoxic effects of the fly ash samples were observed. The increased cytotoxicity may be due to condensation of organics or volatile trace elements on the fly ash surface. When the biological activity vs. the particle size of the fly ash samples (Figure 8) was plotted, cytotoxicity correlated better with the particle size. In this case, the cytotoxicity of the fly ash

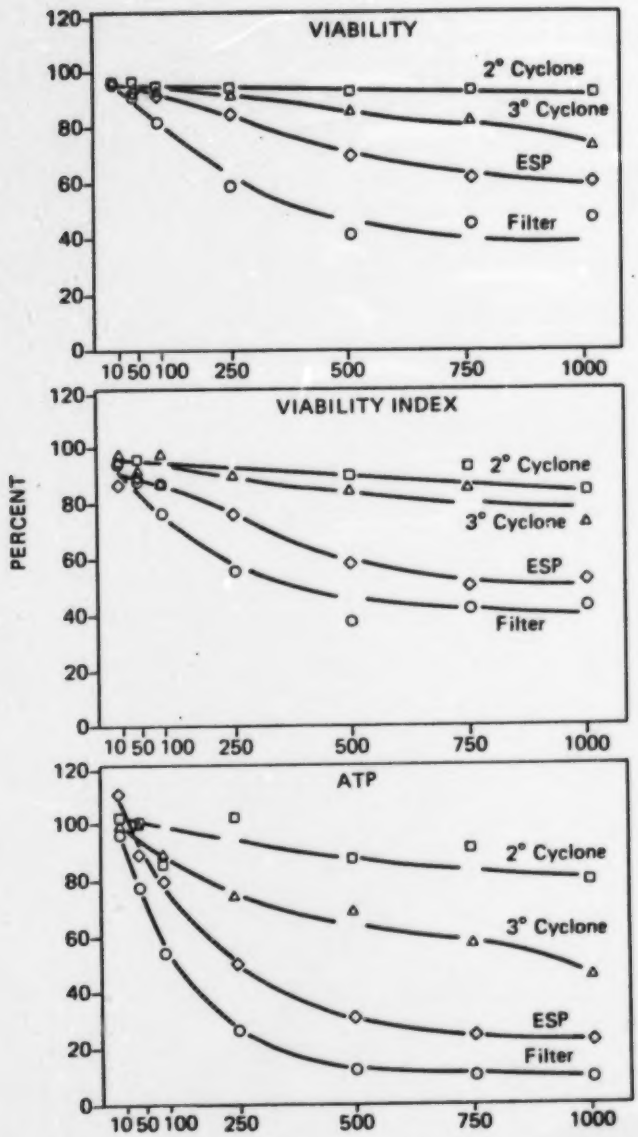

Figure 5. Toxic effects of fly ash on RAM cells after a 20-h exposure in vitro. Data presented are means of six replicates. Viability is calculated and expressed as percent viable cells. Viability index was obtained by multiplying cell viability by total cell numbers as a fraction of the control. Adenosine triphosphate, ATP (fg/ml), is expressed as percent control.

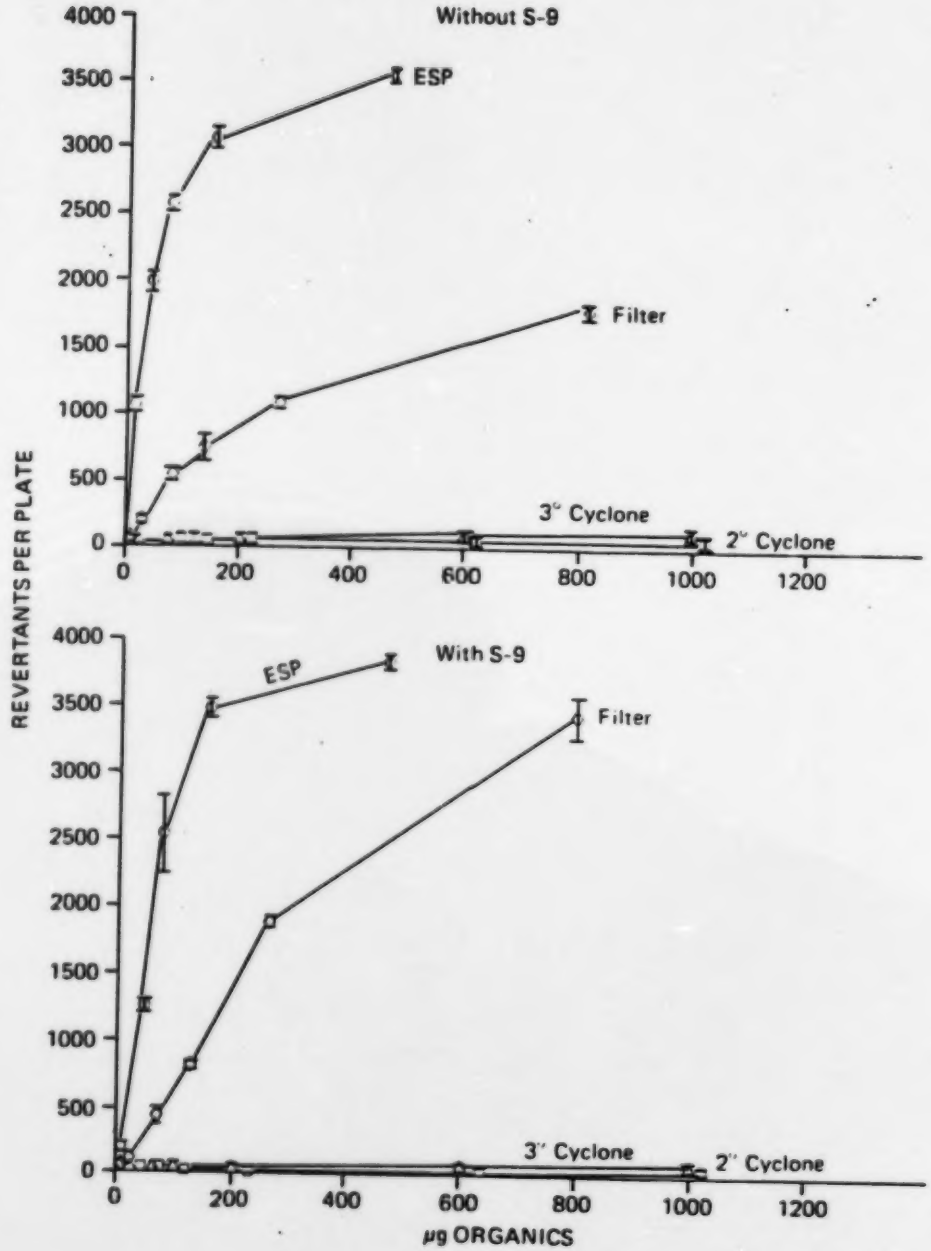

Figure 6. Mutagenicity of dichloromethane extracts of fly ash in Salmonella typhimurium TA98. Data presented are means ± standard deviation of three replicates.

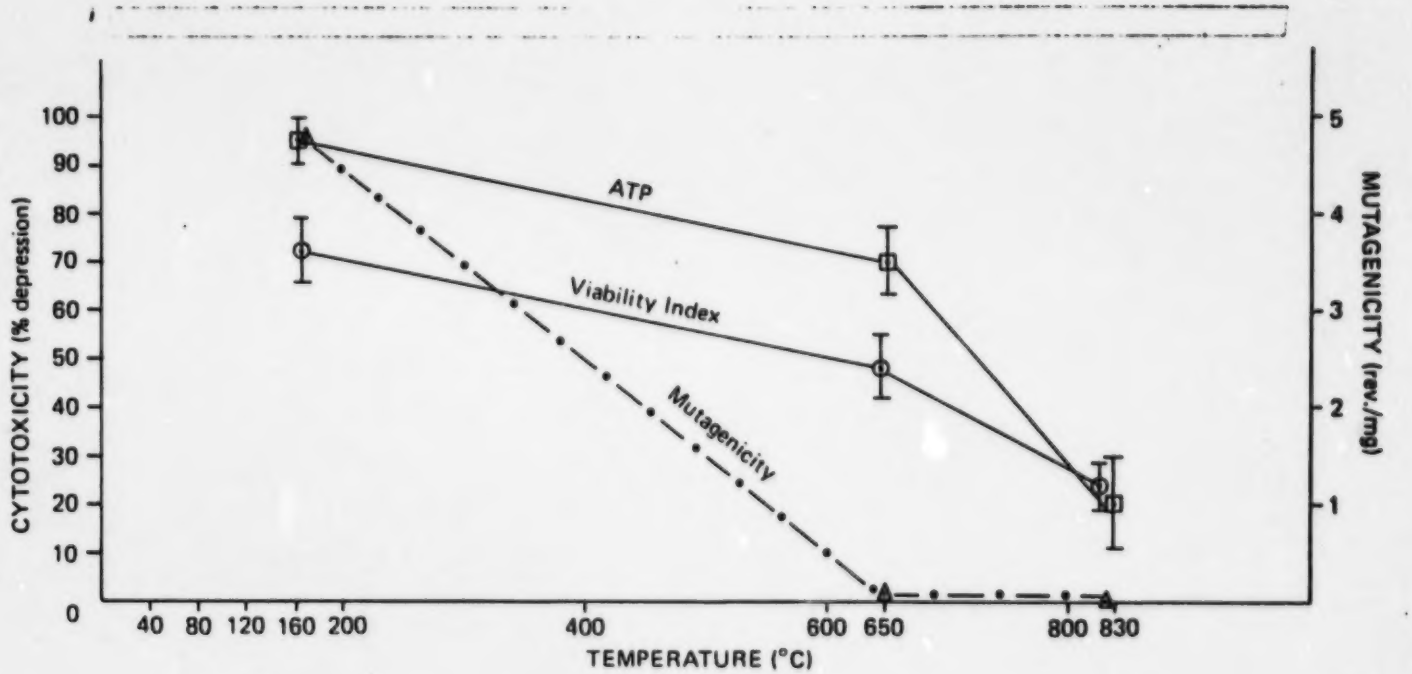

Figure 7. Fly ash biological activity vs. collection temperature. Cytotoxicity data were obtained from experiments using RAM cells exposed for 20 h to 1000 µg/ml of the fly ash samples. Data points are mean ± standard deviation of six replicates. Mutagenicity data were obtained from specific activity (rev/mg) of fly ash samples in Salmonella typhimurium TA98 without S9.

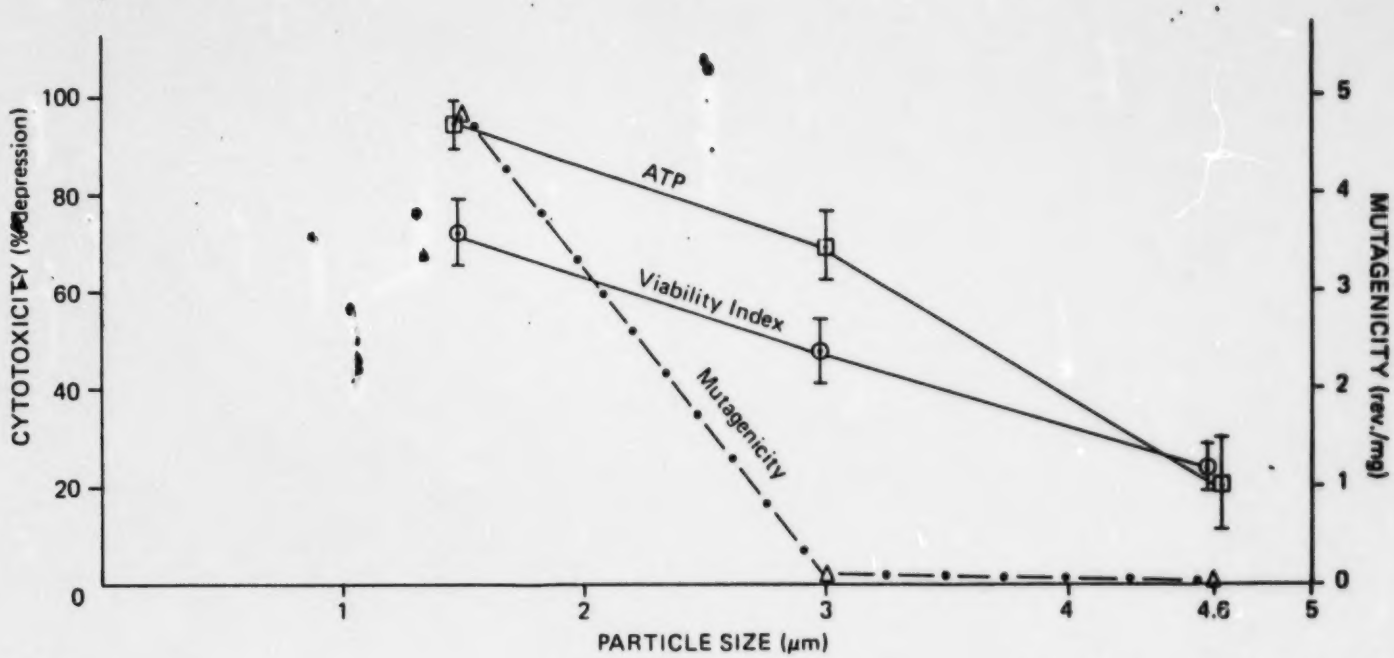

Figure 8. Fly ash biological activity vs. particle size. Cytotoxicity data were obtained from experiments using RAM cells exposed for 20 h to 1000 μg/ml of the fly ash samples. Data points are means ± standard deviation of six replicates. Mutagenicity data were obtained from specific activity (rev/mg) of fly ash samples in Salmonella typhimurium TA98 without S9.

appeared to be more size-dependent than temperature-dependent. The smaller particles have a larger specific area and can adsorb more potentially toxic trace elements and organics on their surface.

Mutagenicity of the samples, however, is more collection-temperature-dependent: The collection temperature must be lowered to a certain point before mutagenic effects are seen. When samples were collected in the high temperature region, mutagenicity did not increase with decreasing particle size. Thermal studies conducted by Fisher et al. (1979) showed that when coal fly ash was heated to 350°C or higher, mutagenicity was completely lost. Thus, the collection temperature, which can affect the degree of adsorption and the kind of organics deposited onto fly ash surface, appears to play an important fole in determining the mutagenicity of the fly ash collected. When the sample is collected at a low temperature, in a range where adsorption of organics occurs, the particle size or specific area of particles will be important in determining the mutagenicity of fly ash.

For investigating the biological effects of coal fly ash, therefore, it is important to collect the samples at cooler temperatures after air dilution, if possible, or to evaluate the vapor phase of the gas effluent downsteam of the particulate collection device when particles are collected at a high temperature. In the Georgetown FBC unit, we examined fly ash samples collected at a fairly high temperature (163°C) and an XAD extract of gas effluent.

The Georgetown University atmospheric FBC boiler is one of the largest commercial applications of fluidized bed coal combustion in this country. Currently, it heats and cools 204,000 m^2 of space in the university. The boiler produces 45,360 kg of steam per hour. Five tons of Eastern bituminous coal containing 3.3% sulfur are burned each hour at ~ 840°C. Limestone is used for the bed material. The primary particulate control devices are mutlicyclones and a baghouse (Young et al., 1981).

Fly ash samples from the Georgetown FBC unit examined were baghouse particulate samples, collected at 163°C, and the vapor phase of the gas effluent, collected by the XAD-2 trap of a Source Assessment Sampling System (SASS) train at 23°C, downstream of the baghouse (Young et al., 1981). The results of the Ames Salmonella mutagenicity assay of these samples are shown in Figure 9. The data show that organic extracts of both gas and particulate effluents are mutagenic in the Salmonella typhimurium assay in strains TA98, TA1538, and TA100, without metabolic activation.

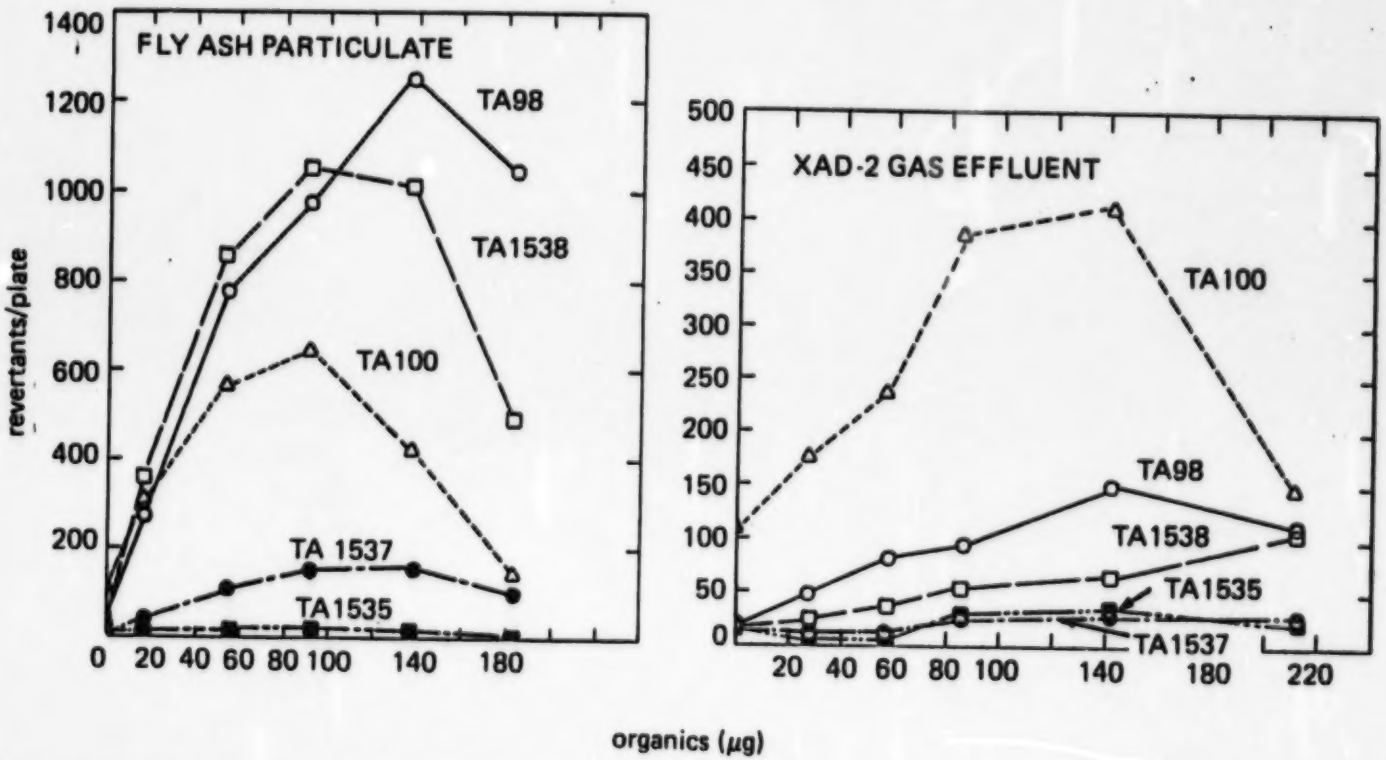

Figure 9. Mutagenicity of the dichloromethane extracts from the gas and particulate effluents of Georgetown FBC in Salmonella typhimurium without S9. Data presented are means ± standard deviation of three replicates.

The organic extract from fly ash was much more mutagenic than the organics obtained from the gas effluent. The mutagenicity of the fly ash extract was 9.26 revertants and 10.0 revertants per microgram of organics in TA98 and TA100, respectively, without activation. The mutagenicity of the extract of the XAD-2 gas effluent sample was 0.96 revertants and 3.92 revertants per microgram of organics in TA98 and TA100, respectively, without activation. The fly ash extract showed comparable high mutagenicity in TA98, TA1538, and TA100, and showed negative response in TA1535. The extract of XAD-2 gas effluent showed much higher mutagenic activity in TA100 than in TA98 or TA1538. Some mutagenic activity was observed in TA1535 for gas effluent. These results suggest that the gas and fly ash effluents may contain different mutagens. The higher activity of the gas effluent in TA100 suggests that it contains both base-pair substitution and frameshift mutagens, whereas the particulate effluent activity in the various tester strains is more consistent with the presence of primarily frameshift mutagens. Addition of S9 for metabolic activation to the fly ash organics generally decreased the mutagenic activity (from 9.26 revertants to 4.65 revertants per microgram of organics in TA98). This indicates that the mutagens in fly ash were direct-acting.

Polycyclic organic matter is known to be emitted from coal combustion. This study showed that the mutagens in coal fly ash are primarily direct-acting and frameshift. Some nitroaromatic compounds have been found to be potent, direct-acting, and frameshift mutagens in the Ames Salmonella assay (Rosenkranz et al., 1980) and are found in combustion products, for instance, diesel emissions (Schuetzle et al., 1981). One nitroaromatic compound, 1-nitropyrene, was detected in the coal combustion products. Table 3 shows the nitropyrene concentration of the particulate and gas phase effluents from the coal combustion sources that was determined by a reverse-phase HPLC fluorescence method (Tejada et al., 1982).

CONCLUSIONS

FBC and CC fly ash particles were found to be cytotoxic in vitro in mammalian lung cells. The organic extracts of FBC and CC fly ash samples contained direct-acting and frameshift mutagens. The direct-acting, frameshift mutagen 1-nitropyrene was detected in the coal combustion products. The fly ash particle size, collection temperature, and sample preparation method were shown to significantly affect the bioassay results. The fly ash particle size, which is affected by the collection site and device, is important in determining the toxicity of fly ash

Table 3. 1-Nitropyrene in Coal Combustion Products

Product	1-Nitropyrene Concentration (ng/g fly ash)	(µg/g organics)
Fly Ash		
FBC--Exxon Miniplant[a]	130	161.1
FBC--Georgetown[b]	0.09	1.7
CC--Power Plant[a]	0.11	0.56
Gas Effluent		
FBC--Georgetown (XAD Extract)		8.0

[a]Respirable particles (< 3 µm).
[b]Larg particles (< 400 µm).

samples collected. The mutagenicity of fly ash can be affected by collection temperature. This finding is further substantiated by the presence of mutagenic activity in the XAD extract of the gas effluent following the collection of the particles at a high temperature. Careful consideration of the above factors is required before any meaningful comparison can be made of the biological data presented by the various investigators.

Fluidized-bed combustion of coal technology is new and still developing. Any improvement in technology such as combustion efficiency can change the biological effects of the fly ash. As the technology develops, fluidized-bed combustion may provide one of the best alternative coal technologies. These studies suggest that monitoring the mutagenicity and cytotoxicity of the emissions during the developmental stage of this technology may help to direct improvements so that the mutagenic and cytotoxic components of the emissions can be minimized.

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